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BY

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INFLUENCE OF BACTERIOPHAGE ON BACTERIUM TUMEFACIENS, AND SOME POTENTIAL STUDIES OF FILTRATES[1]

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INTRODUCTION

While making potentiometer studies of the juices of 50 different species of healthy plants as compared with the juices of tumors produced on the plants by *Bacterium tumefaciens* Sm. and Town., the junior writer was impressed by the frequent reversal of the relative magnitudes of the pH values in the various juices after they had oxidized from one to five days. It was observed that whereas the juice of the normal sugar beet immediately after crushing might be pH 5.9 and that of the crushed tumor 6.3, in two days the juice of the normal sugar beet would be 6.2 and that of the tumor 4.8. It occurred to her that this oxidized tumor juice might influence the organism producing the tumor and that by growing the organism in a suitable medium, along with the oxidized tumor juice which had reversed its pH relative to the pH of the normal juice, there might be obtained a culture that had lost its power to infect.

Two cultures of *Bacterium tumefaciens* were accordingly exposed to different dilutions of the oxidized juice from crushed Ricinus tumors, and after the cultures had been incubated for a few days young Ricinus plants were inoculated with them. At the same time other Ricinus plants were inoculated with control cultures. Although neither treated culture became inactivated as was expected, one produced more rapidly forming and larger tumors and the other more slowly forming and smaller tumors than the controls. Recognizing that something besides oxidation with accompanying pH change had entered into these results, and believing that it was the principle of bacteriophagy, the junior writer began the work which developed into the joint investigation here reported.

REVIEW OF LITERATURE

The isolation from diseased plants of a lytic and inhibiting principle, called by D'Hérelle (*3*)[2] the bacteriophage, has not been carried on so extensively by plant investigators as by animal research workers. The earliest plant investigators who succeeded in isolating lytic principles from nodules of leguminous plants, from roots and stems of legumes, and from garden and field soil were Gerretsen, Gryns, Sack, and Söhngen (*2*). Mallmann and Hemstreet (*7*) isolated an inhibiting substance from a rotted cabbage, but did not demonstrate actual lysis. Coons and Kotila (*1*) isolated from rotted carrot, river water,

[1] Received for publication Mar. 13, 1929; issued October, 1929.
[2] Reference is made by number (italic) to "Literature cited," p. 529.

and soil the bacteriophage which produced inhibition of growth of various bacteria when used in high dilutions and definite lysis when used in stronger concentrations.

In Russia, Israilsky (Izrailsky) (*5*, *6*) demonstrated the bacteriophage in plant tumors produced by *Bacterium tumefaciens*. From nine different isolations from tumors on sugar beets he succeeded in getting two strains which were dissolved by the bacteriophage. In his later work (*6*) he studied the physical-chemical nature of the normal-plant and tumor juice along with that of *Bact. tumefaciens* in culture and concluded that the disappearance of *Bact. tumefaciens* from plant galls must be attributed to the action of a bacteriophage and not to the formation of acid in the galls. He also treated roots, stems, and seeds with the bacteriophage before inoculating them with *Bact. tumefaciens* and found that the bacteriophage reduced appreciably the percentage of infection. He stated, however, that a great many more experiments were necessary before the prophylactic action of the bacteriophage is unquestionably established.

PRELIMINARY EXPERIMENTS

In the preliminary plant inoculations made by the junior writer tumors of two distinct types were produced—one more rapidly growing and larger in size than the control tumors, and the other slow in appearing and smaller in size than the controls. The cultures were made as follows:

Good-sized Ricinus tumors were crushed well and the juice was extracted and allowed to oxidize three days, in which time the pH changed from 5.6 to 5.0. The juice was filtered through paper, and dilutions in beef bouillon of pH 7.0 were carried to about 1 : 1,400 and 1 : 28,000. The tubes were seeded with a 24-hour-old culture of *Bacterium tumefaciens* (hop strain) and left to grow two days. From these dilutions transfers were made to potato-dextrose agar, and Ricinus plants were inoculated with them two days later. Other Ricinus plants of the same age were inoculated with a potato-dextrose agar control culture 2 days old.

In 10 days the inoculations with the 1 : 1,400 dilution culture showed no indication of outgrowths, those with the control cultures had produced swellings 2 to 3 mm. in diameter, and those with the 1 : 28,000 dilution culture had produced little tumors 5 to 6 mm. in diameter. In 19 days very tiny swellings were showing from the inoculations made with the 1 : 1,400 dilution (fig. 1, A), whereas the inoculations made with the 1 : 28,000 dilution (fig. 1, B) had produced tumors twice as large as the controls, (fig. 1, C).

This comparative rate of growth kept up for over a month, the differences being quite marked. In one and one-half months the rapidly growing tumors had reached their limit, 6 cm. in diameter. This was twice the size of the controls. The retarded tumors began to grow rapidly one month after inoculation, but in two months after inoculation the largest of them was only 1.5 cm. in diameter. Very little development took place after that.

TECHNIC

Filtrates of Ricinus tumors and of sugar-beet tumors produced with the hop strain of *Bacterium tumefaciens* were added to fresh beef-bouillon cultures of the hop strain, the 2, 10, and 30 drop method

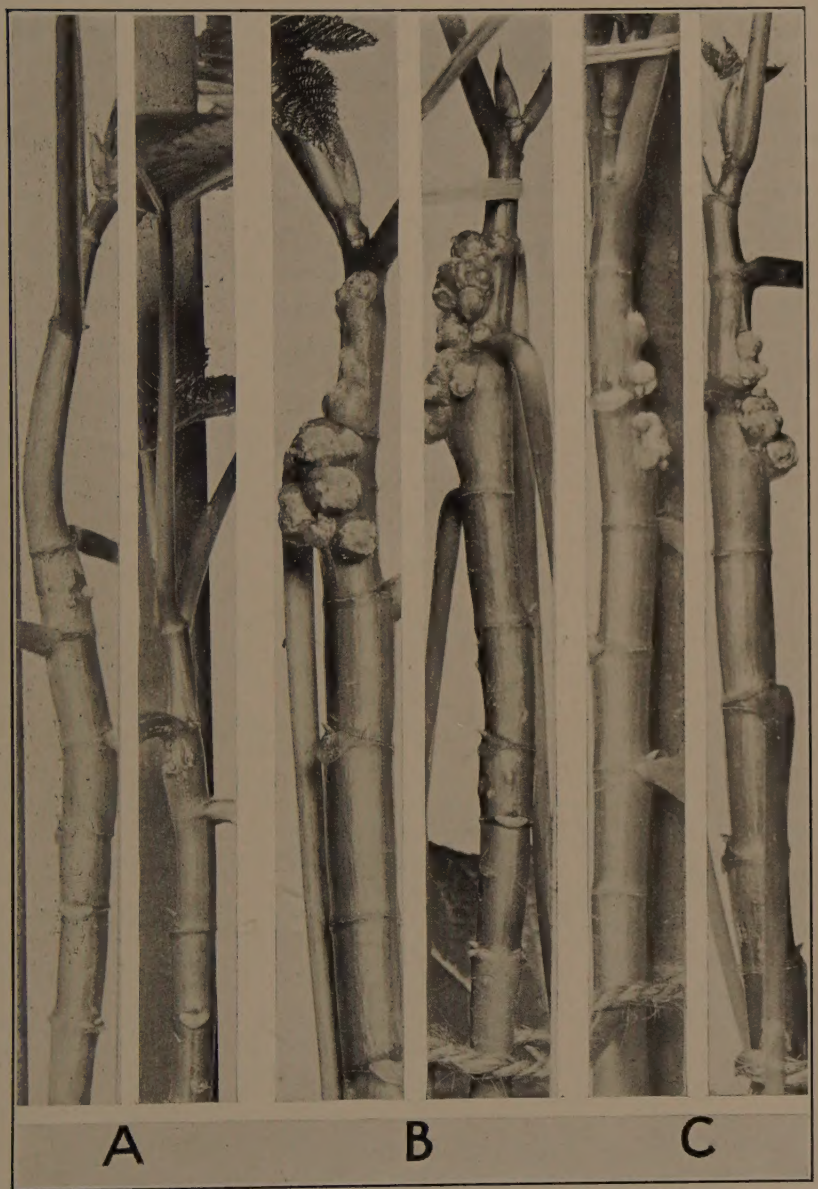

FIGURE 1.—Accelerated and retarded tumors on Ricinus. Plants inoculated January 21, 1927; photographed after 19 days. All natural size. A, Retarded and undersized tumors produced by *Bacterium tumefaciens* (hop strain) plus a low dilution of tumor juice; B, accelerated and oversized tumors produced by *Bact. tumefaciens* plus a higher dilution of tumor juice; C, normal and regular-sized tumors produced by a control (normal) culture of *Bact. tumefaciens*

of D'Hérelle (*3*) being used. These drop cultures were watched for inhibition and lysis. Several 30-drop cultures were usually made, one of which was used to make slant cultures and poured plates for the study of bacteriophage plaques. One of the 30-drop filtrate tubes had a little heavier seeding of the organism than the others, which received only a loop transfer from the young culture. The transfers to the beef bouillon were made from an 18 to 24 hour beef-bouillon culture faintly clouded or from an agar slant of the same age suspended in beef bouillon. The former was found more satisfactory, as the hop strain produces bacterial clumps, a feature not wanted in this type of work. Infusion-beef agar, pH 7.0 to 7.2, was found satisfactory for demonstrating the plaques on the plates. Beef-bouillon media of pH 6.5 to 8.3 were tried for demonstrating inhibition in the seeded filtrate cultures, but those of pH 6.7 to 6.9 were found more satisfactory. Incubation was at temperatures of 22° to 28° C.

Plates were poured from the filtrates to test their sterility, and if they proved not to be sterile this fact was noted.

When plates were poured from the seeded filtrate for the study of plaques, plates of the control cultures were also poured for comparison. Slant cultures were often made from the 2 and 10 drop seeded filtrate cultures as well as from the 30-drop ones. To seed the slants and plates, drops of cultures were carried to them in sterile pipettes, although a sterile cotton swab was sometimes used for this purpose. The seeded poured plate was found the most satisfactory of all methods to demonstrate the plaques.

Except in the case of the first experiment which demonstrated the acceleration and retardation of the growth of tumors, the juices were filtered. Chamberland L3 filters were used after the juice had first been put through filter paper or centrifuged. If one of the 30-drop seeded filtrate cultures showed inhibition or slight growth in 24 hours it was refiltered and the organism exposed again to the new filtrate. These passages of the organism with the filtrate through successive trials were carried along to increase the potency of the inhibiting substance present, in the hope that lysis would be attained. Sometimes only one and at other times several filterings and seedings were made in eight hours.

Dilutions of the tumor filtrates from 1:10 to 1:10,000,000,000 in tubes with beef bouillon seeded with the organism were also used for the study of inhibition and lysis. Control cultures were always held in the same racks for comparison.

Filtrates of normal Ricinus juice, of carrots rotted with *Bacillus carotovorus*, of normal carrots, and of sewage were also tested with the organism by both the drop and the high-dilution methods.

The strains of *Bacterium tumefaciens* tested were hop, peach, and daisy.

Plants of *Ricinus communis* (castor-oil plant) were used for the inoculations because they grow fast in the greenhouse and produce tumors quickly. The hop strain of *Bacterium tumefaciens*, which was used for most of the work, produces 100 per cent infection on Ricinus.

PHYSICAL-CHEMICAL ASPECTS OF THE FILTRATES

The study of *Bacterium tumefaciens* in culture and experiments on plants with chemical substances produced by *Bact. tumefaciens*, together with experiments limiting the intake of oxygen and so compelling the cells to manufacture the stimulus which leads to the development of hyperplasias, led Smith (*9*) to state that—

> All tumors, so far as they are due to parasites, must be assumed to be due to the chemical-physical action of the by-products of the metabolism of these parasites, just as most communicable diseases are due not to the parasites themselves, but to their toxins. * * * Diverse as are the tumors of plants due to parasites, I think that the fundamental chemical-physical phenomena underlying them are much alike, and that the differences we see, when these differences are not due wholly to varying tissue reactions, must result simply from variations in volume, and direction and continuity of the chemical-physical stimulus.

Since these statements were published, much has been added to confirm Smith's conclusions by the more direct pH, total-acid, and oxidation-potential measurements and dilution experiments on the juices of normal and tumor tissues. The striking facts revealed by these later studies are: (1) The pH of the freshly extracted juice of tumor tissue was always higher or the reaction more alkaline than that of the normal juice; (2) the titratable-acid content was greater in the tumor than in the normal juice; (3) the oxidation potential was always greater (more negative) in the freshly extracted tumor juice than in the normal juice—that is, the electrical charge carried by the gold electrode in the tumor juice was always negative (−) to that in the normal juice; (4) the electrical charges (accompanied always by a change in pH) on the gold electrodes were exactly reversed in the normal and tumor-tissue juices upon further oxidation; and (5) the change in pH produced by the growth of the organism (*Bacterium tumefaciens*) in beef bouillon cultures and the change in pH produced by *Bacillus carotovorus* when inoculated into fresh carrots progressed in the same direction (increase) as the change of pH of the juice of the normal tissue on further oxidation, rather than in the direction (decrease) of the pH changes of the juice of the tumor tissue.

The juices of the tumor are always more colloidal than the juices of normal tissue, as evidenced by the degree of filterability. The influencing factor is not precipitated by centrifugation and is active in high dilutions as an accelerator of *Bacterium tumefaciens* and in low dilutions as an inhibitory factor.

Since the substance in the plant filtrates which in the presence of molecular oxygen are apparently associated with oxidation and reduction phenomena causing changes in the pH and their oxidation potentials (with gold electrodes) behaved the same in all plants studied, and were consistently linked with the normal and tumor-tissue juices, it was thought that such potential determinations might give evidence when bacteriophage plaques could be expected on poured plates. That there was some justification for this belief is brought out by a study of Table 1 and the poured-plate and plant-inoculation experiments given later.

TABLE 1.—*Titratable-acid, pH, and oxidation-potential measurements* [a] *of different filtrates and the plaques on poured agar plates resulting from treating the cultures of Bacterium tumefaciens (hop strain) with them*

Filtrates and cultures tested	Freshly extracted filtrates and young cultures				Oxidized filtrates and old cultures		Plaques
	Initial pH	N/1 alkali required to increase pH to 8.2	Oxidation potential differences	Charge (plus or minus)	Initial pH	Charge (plus or minus)	
		C. c.	*Millivolts*				
Ricinus with mature tumor:							
Tumor	5.5	22.5	125.6	−	4.9	+	Present.
Normal	5.4	16		+	6.8	−	
Ricinus with rotted tumor:							
Tumor	6.8		103.0	−	7.1	−	Absent.
Normal	5.4			+	8.2	−	
Ricinus with dried tumor plus water:							
Tumor	6.8			−	6.8	−	Very few present.
Normal	5.5			+	8.0	−	
Sugar beet with mature tumor:							
Tumor	6.2	23.5	125.0	−	5.0	+	Present.
Normal	5.9	18		+	6.4	−	
Sugar beet with older, mature tumor:							
Tumor	6.8	25	143.2	−	5.1	+	Do.
Normal	6.3	19.5		+	6.8	−	
Carrot:							
Normal, fresh and young	6.8						
13-day-old normal, young					7.1		
Inoculated 70 days with Bacillus carotovorus.					9.6		
Bacterium tumefaciens:							
Plus juice of carrot infected with B. carotovorus; culture 1 day old.	[b] 7.1						Many present.
Plus juice of carrot infected with B. carotovorus; culture 4 days old.					6.6		
24-hour-old normal culture	6.7						Present.
1-month-old normal culture					8.2		Absent.

[a] The followingprocedure was suggested by S. F. Acree of the Bureau of Standards: Two batteries, consisting each of a saturated calomel electrode and a Hildebrand cell, were joined by a platinum wire, connecting the two calomel cells. Each Hildebrand cell contained a gold or a hydrogen electrode as desired. Usually 10 c. c. of the normal or tumor juice was used for each test. This equipment made it possible (1) to measure both the hydrogen-ion and the oxidation potentials against a standard calomel cell, or (2) to obtain directly the differences in these potentials shown by the normal and tumor extracts. The latter method was used generally with occasional checks of each sample against the calomel electrode with a type K Leeds & Northrup potentiometer. The estimation of potentials was made on expressed juice of normal and tumor tissue. The same volume of normal and tumor juice from the same plant was used for comparison in each experiment. The same environmental conditions as to temperature, gases, technic, etc., made the results more accurate as they are dependent on the simultaneous handling of both samples.

[b] This culture was split and tested 4 days later, but no plates were poured on the fourth day.

It is shown in Table 1 that the freshly extracted juice from Ricinus and sugar beet gave pH values of 5.4 to 6.3 and carried a positive (+) charge when compared to the juice of the tumor; the same juice oxidized from two to five days gave pH values progressing in the alkaline direction accompanied by a minus (−) charge. The opposite relationship of values, both as to pH and to charge carried, to those of the normal juice was obtained on the freshly extracted and oxidized tumor juice. The freshly extracted juice of a mature tumor was more alkaline than the normal juice in its initial pH values and carried a minus (−) charge, but upon oxidation for two to five days the pH values became distinctly more acid with an accompanying change of electrical charge (+). *Bacterium tumefaciens* treated with this filtrate showed numerous plaques on the poured plates. In the case of the rotted tumor juice the pH value proceeded in an alkaline direction, and in that of the dried tumor juice plus water the value remained about

stationary. It is a striking fact that from the last two filtrates, where the pH progressed in the alkaline direction or remained stationary, few or no plaques were found on the poured plates. The total acid content was found to be greater in the freshly extracted tumor juice than in the normal juice; also a definite oxidation-potential value in favor of the freshly extracted tumor juice above that of the normal juice was registered. The reversal of pH ceased at about pH 5.1 to 4.9 in the juices of the tumors from Ricinus and sugar beet.

The freshly extracted juice of young carrots gave pH values of 6.8, whereas the same juice left standing 13 days gave pH 7.1. A *Bacterium tumefaciens* culture treated with the fresh filtrate of normal carrot produced plaques. Juice of carrots 70 days after inoculation with *Bacillus carotovorus* gave pH 9.6. The pH direction taken by carrot inoculated with *B. carotovorus* is the same as that taken by *Bact. tumefaciens* in beef-infusion medium and that of the normal-carrot juice upon further oxidation.

A freshly seeded *Bacterium tumefaciens* culture treated with the filtrate of carrots inoculated with *Bacillus carotovorus* gave a value of pH 7.1. With this filtrate the plaques on poured plates were more numerous than with any of the other filtrates. This same culture held four days gave pH 6.6. Here again the pH is in the more acid direction when compared with the values of the juice from carrot inoculated with *B. carotovorus* (pH 9.6) and *Bact. tumefaciens* culture 1 day old (pH 6.7) or 1 month old (pH 8.2). At two different times a 24-hour culture of *Bact. tumefaciens*, pH 6.7, gave good plaques on poured plates. These were exceptions rather than the rule. For the range of growth of *Bact. tumefaciens* in beef-infusion medium an earlier paper (*8*) may be consulted.

Three Ricinus plants were inoculated with each of the tenfold dilution cultures when the cultures were two days old, and three other Ricinus plants were inoculated with a control culture of the same age. In nine days many of the little tumors resulting from the dilution inoculations were growing faster than the control tumors. In less than a month the tumors forming on 17 of the plants inoculated with the dilution culture were larger than the control-culture tumors, 8 were of the same size, and only 5 were slightly smaller. All of the sixth-dilution (1:1,000,000) inoculations produced smaller tumors than the controls, while all the first, fifth, eighth, and two each of the seventh and tenth dilutions produced larger tumors.

To determine whether inhibition might occur if larger quantities of the same filtrate were used in the bouillon, a series of dilutions was made with 9 parts of the filtrate to 1 part of beef bouillon. To a tube of 1 c. c. of beef bouillon 9 c. c. of filtrate was added, thus making a 9:10 dilution. Ten dilutions were made as before, the last being 9:1,000,000,000. Each was seeded with a loop of a 24-hour culture of the hop strain. Heavier growth than in the controls took place in 24 hours in all cultures except the 9:10, which had none. A heavy pellicle formed in all except the 9:10. In 48 hours, however, this culture also had what appeared to be a trace of growth. Inoculations with these 11 cultures (including the control) were made the second day into each of three Ricinus plants.

Practically the same relationship of acceleration of tumor growth and increased size of tumors over the controls was observed in the

9:10 series as in the 1:10 series. Inoculations with the first dilution, 9:10, did not produce tumors. Table 2 gives the results of the inoculations with both series.

TABLE 2.—*Results of inoculating Ricinus plants with the hop strain grown two days with filtrates of Ricinus tumors in dilutions of 1:10 to 1:10,000,000,000 and 9:10 to 9:1,000,000,000, respectively*

Dilution and period between inoculation and examination	Dilutions of 1:10 to 1:10,000,000,000				Dilutions of 9:10 to 9:1,000,000,000			
	Initial pH	Diameter of tumors on—			Initial pH	Diameter of tumors on—		
		Plant No. 1	Plant No. 2	Plant No. 3		Plant No. 1	Plant No. 2	Plant No. 3
		Mm.	*Mm.*	*Mm.*		*Mm.*	*Mm.*	*Mm.*
No. 1:								
9 days	6.8	9–12	9–12	9–12	5.3	0	0	0
1 month		20–30	20–30	20–30		0	0	0
No. 2:								
9 days	7.1	9–12	0	0	5.4	9–12	9–12	9–12
1 month		30–40	13–20	9–12		20–30	20–30	20–30
No. 3:								
9 days	6.8	9–12	9–12	9–12	7.0	9–12	9–12	6–8
1 month		30–40	13–20	13–20		20–30	20–30	9–12
No. 4:								
9 days	6.7	9–12	9–12	9–12	7.0	9–12	0	0
1 month		20–30	13–20	9–12		13–20	13–20	9–12
No. 5:								
9 days	6.9	9–12	9–12	9–12	7.1	9–12	9–12	6–8
1 month		20–30	20–30	20–30		20–30	9–12	9–12
No. 6:								
9 days	7.3	9–12	4–5	4–5	7.1	9–12	0	0
1 month		9–12	9–12	9–12		9–12	9–12	9–12
No. 7:								
9 days	6.9	9–12	0	0	6.9	9–12	9–12	6–8
1 month		20–30	20–30	13–20		9–12	9–12	20–30
No. 8:								
9 days	6.7	9–12	0	0	6.9	9–12	9–12	0
1 month		20–30	20–30	20–30		20–30	20–30	9–12
No. 9:								
9 days	7.1	9–12	0	0	7.1	9–12	0	6–8
1 month		20–30	13–20	13–20		20–30	20–30	9–12
No. 10:								
9 days	7.0	9–12	9–12	9–12	7.3	9–12	0	0
1 month		30–40	30–40	13–20		20–30	20–30	20–30
Control: [a]								
9 days	7.8	4–5	0	0	7.8	4–5	0	0
1 month		13–20	13–20	13–20		13–20	13–20	13–20

[a] Same control plants used for both dilution series.

EFFECTS OF DIFFERENT TREATMENTS ON THE HOP STRAIN

EFFECTS OF DILUTE FILTRATE OF RICINUS TUMORS ON PRODUCTION OF TUMOR JUICE FILTERED AFTER 3-DAY OXIDATION

Filtrates of Ricinus tumor which were found to be sterile by the poured-plate method after passing through Chamberland L3 filters were studies in dilution experiments. The juice had been exposed to the air for three days before it was filtered and its pH had changed from 5.5 to 4.9. A series of tubes containing 9 c. c. of infusion-beef bouillon, pH 6.9, was treated as follows: With a sterile pipette 1 c. c. of a filtrate of a Ricinus tumor was added to the first tube and mixed thoroughly by shaking. This gave a dilution of 1:10. With another sterile pipette 1 c. c. of this dilution was added to the next tube, making the second a 1:100 dilution. The method was repeated, each tube was shaken and a sterile pipette was used for each of the

10 tubes of the series until a dilution of 1:10,000,000,000 was reached. The dilutions were each seeded with a loop of a 24-hour bouillon culture of the hop strain, for in a previous test a drop of the culture had been found to be too heavy.

In 18 to 24 hours growth was heavier than in the controls in all but the 1:10 dilution. In 2 days there was also growth in the 1:10 dilution.

Juice Filtered Immediately

The dilution series 1:10,000,000,000 was repeated with two other filtrates (pH 5.8 and 5.5) from Ricinus tumors. As unoxidized juice was desired for this experiment, both sets were filtered, diluted in series, and seeded with the hop strain the same day the tumors were cut. No retardation of growth was observed in the tubes of either set except in the 1:10 dilutions, which lasted for only about 24 hours. Both sets were well clouded in 48 hours. The other nine dilutions showed greater growth than the controls in 24 hours.

Two Ricinus plants were inoculated with each of the 10 dilutions of the second of the unoxidized sets. Two inoculations with control cultures were also made. The tumors grew more rapidly and developed to greater size in the fifth, sixth, seventh, and eighth dilution inoculations than in the controls; the tumors in the ninth and tenth dilution inoculations were the same size as control tumors; while the tumors in the first, second, third, and fourth dilution inoculations within a little more than a month were slightly smaller than those of the controls.

From these tests it appears that tumor juices filtered and used immediately and those oxidized several days before filtering and used in high dilutions with the organism have power to produce more rapidly growing and larger tumors than the control cultures; they also have power to produce larger tumors than the controls when they are inoculated into susceptible plants.

Comparative Effects of Filtrates of Bacterium tumefaciens, Normal Ricinus Plants, and Necrosed Ricinus Tumors

A 24-hour beef-bouillon flask culture of *Bacterium tumefaciens* was filtered, the filtrate was carried in tubes in dilutions from 1:10 to 1:10,000,000,000, and each dilution was inoculated with *Bact. tumefaciens* (hop strain) to see how the organism would react with a culture filtrate of itself added. In 24 hours there was slight growth in the controls but no growth in any of the dilutions. In 48 hours, however, there was growth in all 10 dilutions, and in many of them it was heavier than in the controls.

A filtrate of normal Ricinus plants and one of necrosed Ricinus tumors were carried each in 10-dilution tubes in the same way, 1:10 to 1:10,000,000,000, and each dilution was inoculated with the hop strain for comparison with the culture-filtrate dilutions. In 24 hours no growth had occurred in these two sets. In 48 hours, however, there was heavier growth in some of the dilutions with filtrate of the normal Ricinus plant than there was in the controls. There was growth equal to the controls in the cultures with necrosed Ricinus tumor filtrate. The pellicles were not so heavy as in the controls in the 1:10 and 1:100 dilutions of the latter.

Inoculations into young Ricinus plants were made when the dilution cultures were 4 days old. The plants were a little younger than is desirable for inoculating and the growing tumors killed a few of them.

Many of the culture-filtrate dilutions produced tumors more rapidly than the control cultures, the normal Ricinus filtrate cultures, or the necrosed tumor filtrate cultures. The tumors were also larger than those produced by the necrosed tumor filtrate inoculations, but were not very different in size from those of the normal Ricinus tumors. Table 3 gives details of this experiment.

EFFECTS OF REPEATED FILTERINGS WITH DIFFERENT FILTRATES

Filtrates of Old and Slightly Necrosed Ricinus Tumors

The pH of the juice of 5-months-old Ricinus tumors was 6.8 before filtering and 6.2 after filtering. The pH of the juice of healthy Ricinus plants of the same age was 5.4.

Two, ten, and thirty drops of the 5-months-old tumor filtrate were added to fresh (hop strain) cultures in beef bouillon (pH 6.7). In 24 hours the growth was slight in the drop cultures but equal to that in the controls. Bacteriophage plaques were present on the drop plates, however, in two days. Refilterings of the drop cultures were made, and the tests with tube cultures and poured plates were carried through the third passage. There was still no inhibition in the third passage, and no bacteriophage plaques occurred in the drop-filtrate or the control plates.

Seven days later more of the 5-months-old Ricinus tumor juice was filtered, and *Bacterium tumefaciens* was exposed to it in the usual way. The filtrate had then changed from pH 6.8 to 7.1. A test with 2, 10, and 30 drops was made in the morning and another in the afternoon, using hop strain in beef bouillon (pH 6.7). In both sets growth of the organism was more rapid in the drop tubes than in the controls. No bacteriophage plaques were present on the drop-filtrate plates. The plates poured to test the sterility of the old tumor filtrate had contaminating but no *Bact. tumefaciens* colonies. To make a further test, the unused part of the 7-day-old tumor filtrate was filtered a second time. The filtrate was sterile, for no contaminating colonies appeared on the plates poured from it. The hop strain was again exposed to the old filtrate by the drop method to see if retardation would occur. There was none.

Table 3.—*Results of inoculating Ricinus plants with Bacterium tumefaciens (hop strain) grown four days in 1:10 to 1:10,000,000,000 dilutions of filtrates of Bacterium tumefaciens (hop strain) of normal Ricinus plants, and of necrosed Ricinus tumors, respectively*

Dilution and period between inoculation and examination	Filtrate of beef-bouillon culture of hop strain (pH 6.7)			Filtrate of normal Ricinus plants (pH 5.4)			Filtrate of necrosed Ricinus tumors (pH 6.8)		
	Initial pH	Diameter of tumors on—		Initial pH	Diameter of tumors on—		Initial pH	Diameter of tumors on—	
		Plant No. 1	Plant No. 2		Plant No. 1	Plant No. 2		Plant No. 1	Plant No. 2
1 to 10:									
10 days	7.8	*Mm.* 9–12	*Mm.* 9–12	7.7	*Mm.* 6–8	*Mm.* 6–8	7.5	*Mm.* 6.8	*Mm.* 6–8
19 days		13–15	13–15		9–12	9–12		13–15	13–15
2½ months		35–45	Dead.		18–28	18–28		15–18	15–18

TABLE 3.—*Results of inoculating Ricinus plants with Bacterium tumefaciens (hop strain) grown four days in 1:10 to 1:10,000,000,000 dilutions of filtrates of Bacterium tumefaciens (hop strain) of normal Ricinus plants, and of necrosed Ricinus tumors, respectively*—Continued

Dilution and period between inoculation and examination.	Filtrate of beef-bouillon culture of hop strain (pH 6.7)			Filtrate of normal Ricinus plants (pH 5.4)			Filtrate of necrosed Ricinus tumors (pH 6.8)		
	Initial pH	Diameter of tumors on—		Initial pH	Diameter of tumors on—		Initial pH	Diameter of tumors on—	
		Plant No. 1	Plant No. 2		Plant No. 1	Plant No. 2		Plant No. 1	Plant No. 2
		Mm.	*Mm.*		*Mm.*	*Mm.*		*Mm.*	*Mm.*
1 to 100:									
10 days	7.7	9–12	9–12	7.7	6–8	6–8	7.5	6–8	6–8
19 days		13–15	13–15		9–12	9–12		13–15	13–15
2½ months		30–35	Dead.		35–45	18–28		15–18	15–18
1 to 1,000:									
10 days	7.5	9–12	9–12	7.2	6–8	6–8	8.1	6–8	6–8
19 days		13–15	13–15		6–8	6–8		9–12	9–12
2½ months		28–30	15–18		35–45	35–45		15–18	Dead.
1 to 10,000:									
10 days	7.5	9–12	9–12	7.6	6–8	6–8	8.1	6–8	6–8
19 days		9–12	9–12		9–12	9–12		13–15	9–12
2½ months		35–45	15–18		35–45	18–28		18–28	18–28
1 to 100,000:									
10 days	7.5	9–12	9–12	7.8	6–8	6–8	8.2	6–8	6–8
19 days		13–15	9–12		13–15	9–12		9–12	6–8
2½ months		35–45	30–35		35–45	18–28		18–28	15–18
1 to 1,000,000:									
10 days	7.5	9–12	9–12	7.7	6–8	6–8	8.2	4–5	4–5
19 days		13–15	13–15		13–15	9–12		9–12	9–12
2½ months		30–35	30–35		18–28	18–28		18–28	18–28
1 to 10,000,000:									
10 days	7.5	9–12	9–12	7.7	6–8	6–8	7.7	4–5	4–5
19 days		13–15	13–15		9–12	9–12		9–12	9–12
2½ months		35–45	35–45		18–28	18–28		15–18	15–18
1 to 100,000,000:									
10 days	7.5	9–12	9–12	7.6	6–8	6–8	8.0	6–8	6–8
19 days		9–12	9–12		9–12	9–12		9–12	9–12
2½ months		35–45	30–35		35–45	35–45		18–28	15–18
1 to 1,000,000,000:									
10 days	7.5	9–12	9–12	7.7	6–8	6–8	7.4	6–8	6–8
19 days		9–12	9–12		13–15	13–15		9–12	6–8
2½ months		18–28	28–30		18–28	Dead.		18–28	9–12
1 to 10,000,000,000:									
10 days	7.5	9–12	9–12	7.7	6–8	4–5	7.7	6–8	6–8
19 days		13–15	9–12		13–15	9–12		9–12	9–12
2½ months		15–18	15–18		18–28	18–28		18–28	15–18
Control:[a]									
10 days	7.3	6–8	6–8	7.3	6–8	6–8	7.3	6–8	6–8
19 days		9–12	9–12		9–12	9–12		9–12	9–12
2½ months		18–28	18–28		18–28	18–28		18–28	18–28

[a] Control culture 4 days old. The same control plant was used for each dilution series.

This time, as the filtrate was sterile, in 24 hours the growth in the filtrate tubes was equal to that in the controls but not greater. It duplicated that in the third-passage tests made when the filtrate was used immediately after the tumors were crushed. No bacteriophage plaques appeared on the plates. The direction of the pH change of the filtrate of this particular Ricinus tumor was to the more alkaline side, which is contrary to that usually found in the younger tumors.

FILTRATES OF OLD DRY RICINUS TUMORS

It was thought that an active bacteriophage might have prevented necrosis in the old Ricinus tumors, for even when 6 to 7 months old they were still sound, although more or less dry. Fifty

tumors were ground up, and the juice was extracted and filtered. The hop strain was exposed to 2, 10, and 30 drops of this filtrate, and still other tests were made with the dry tumors. Some of the mashings were left in extract and infusion-beef bouillon for 5 and 24 hours, respectively. Each solution was filtered, and the hop strain was again exposed to 2, 10, and 30 drops of the different filtrates.

None of the filtrates of the dry tumor showed inhibition, and the bacteriophage plaques on the plates accompanying each test were very few and small or there were none at all. In each test there was refiltering with accompanying exposure of the hop strain, but still no inhibition or acceleration of growth as compared to that of the controls was noted.

Filtrates of Sugar-Beet tumors

Tumors on the sugar beet were produced with the hop strain of *Bacterium tumefaciens*. They were crushed, the juice was filtered and 2, 10, and 30 drops of the filtrate were added to fresh beef-bouillon transfers of *Bact. tumefaciens*. Growth in the 2 and 10 drop cultures in 24 hours was heavier than in the control, while that in the 30-drop culture was retarded. In order to speed up the potency of the inhibiting factor, one of the 30-drop cultures was filtered and 2, 10, and 30 drops of this were added to a fresh beef-bouillon culture of *Bact. tumefaciens*. These filterings were carried through five passages, and while plaques appeared on the agar plates and slants, indicating the presence of the bacteriophage, the inhibition in the cultures lasted only 2 to 3 days. Hardened agar plates streaked with cultures made from several of the refiltered and inoculated cultures also showed the bacteriophage plaques.

Some of the plaques were transferred to fresh beef-bouillon cultures of *Bacterium tumefaciens*, but no difference was noted between the plaque-containing culture and the controls. The bacteriophage did not seem to be potent enough to cause lysis or even to retard cultures.

Ricinus plants were inoculated with the 10-drop and with one of the two 30-drop cultures of the original filtrate and organism. These inoculations produced tumors somewhat more rapidly than the controls and for about three weeks they were larger. In one month, however, the tumors in the controls equaled them in size.

A filtrate of other sugar-beet tumors (hop strain), oxidized for 12 days, in which the pH had changed from 6.2 to 5.0, was also used with *Bacterium tumefaciens*. Ten drops of the filtrate added to a transfer from a 24-hour culture of the hop strain produced a culture that had an unusual appearance. In the 10 days that it was under observation no pellicle formed, but there was a heavy precipitate in the bottom of the tube. The precipitate was not viscid as such precipitates usually are, and there was a heavy suspension of growth throughout the culture. Inoculations into Ricinus plants were made with the culture when it was 10 days old. The inhibiting factor evidently was present, for none of the Ricinus plants inoculated showed any outgrowths until after 5 weeks; then tumors appeared and developed very slowly. In 2 months they were only 1 cm. in diameter, while the control tumors were 4 cm. in diameter.

SEWAGE FILTRATE

A sewage filtrate was also used in the 2, 10, and 30 drop method with the hop strain. There were many refilterings, and the filtrate was added each time to a freshly seeded beef-bouillon culture. Retardation of growth did not occur until the third passage, and it did not last 48 hours. Filterings were continued with the use of one of the bacteriophage plaques from the third-passage plates in culture with the organism. The passages which followed produced more of the bacteriophage plaques on plates and slants, but retardation in the tube cultures was about the same. In the fifth passage of the plaque filterings growth was more rapid in the tube cultures than in the controls, and the plaques on the plates practically disappeared.

To ascertain whether this acceleration of growth in the tubes could be demonstrated in tumors on plants, Ricinus plants were inoculated with the 10 and 30 drop cultures. At the same time other Ricinus plants of the same age were inoculated with control cultures, and still others with 10 and 30 drops of sugar-beet tumor filtrate grown with the hop strain. In a week the sewage-filtrate hop inoculations showed well-defined outgrowths 4 mm. in diameter, the beet-filtrate hop inoculations showed good swellings though smaller, while the control-culture inoculations showed mere surface swellings. These differences continued for nearly three weeks, but in four weeks the control and the beet-filtrate tumors had nearly reached the size of the sewage-filtrate tumors.

EFFECTS ON THE DAISY STRAIN OF REPEATED FILTERINGS WITH FILTRATES OF SUGAR-BEET TUMORS

The attempt to isolate an inhibitory substance from filtrates of sugar-beet tumor was continued with these filtrates in company with the daisy strain of *Bacterium tumefaciens*. A feature of this strain is that it does not grow so rapidly on beef agar and in beef bouillon as does the hop strain.

In the first two passages of the sugar-beet tumor filtrate and the daisy strain there was greater growth than in the controls, and no bacteriophage plaques appeared on the plates. Slight inhibition began in the third passage.

Marked inhibition of growth in the 2, 10, 30, and 60 drop filtrate tubes inoculated with the daisy strain occurred, and bacteriophage plaques appeared on the plates of the fourth passage. A plaque from one of these plates was transferred to a slightly clouded beef-bouillon culture of daisy strain, incubated, filtered, and further passages made with the daisy strain, sometimes two and three and at other times only one in eight hours. This was continued through 24 passages. The activity of this filtrate embracing the bacteriophage plaque was marked beginning with the second passage, for there was inhibition of growth in the tube cultures, and plaques appeared on the plates from the second through the eighteenth passage. At this stage an older agar suspension was used instead of the usual 18 to 24 hour culture. With this older culture the activity of the bacteriophage seemingly was submerged, for no plaques appeared on the plates from the nineteenth to the twenty-fourth passage, when the experiment was discontinued. Neither was there a trace of inhibition of growth

in the 2, 10, 30, and 60 drop cultures of the twentieth to the twenty-fourth passage, and only slight inhibition in the nineteenth.

Partial lysis occurred in the tenth passage. Inhibition of growth continued up to the twelfth day in the 30-drop tube, then growth began, but there was only faint clouding with no formation of pellicle. Dead bacteria were in a precipitate at the bottom of the tube, and there was no motion in those alive in the suspended medium when examined in hanging drops.

Ricinus plants were inoculated with a portion of the tenth-passage inhibited culture, and other Ricinus plants were inoculated with a control culture of the same age. Tumors appeared on the control plants, but none on those inoculated with the inhibited culture. Examination of plants was made as late as two and one-half months after the inoculations.

The eleventh passage of the daisy strain with the sugar-beet tumor also showed inhibition in the 30-drop culture. Cowpeas were inoculated with this culture after growth took place, but no tumors resulted. The control inoculations gave small tumors.

Inhibition was not so marked in the twelfth to the eighteenth passages.

Some of the inhibited tenth-passage culture was filtered and treated in a series of new passages with the daisy strain. Much was expected of this filtrate, but no trace of inhibition was shown in two passages. This may have been due to the fact that an older culture of daisy was used for the initial passages, as an 18 to 24 hour culture was not available. It is quite essential to use a 24-hour or less beef-bouillon or agar culture suspension in beef bouillon.

EFFECTS OF FILTRATES OF ROTTED CARROTS ON THE HOP STRAIN OF BACTERIUM TUMEFACIENS AND OTHER ORGANISMS

When *Bacterium tumefaciens* (hop strain) was exposed to rotted-carrot filtrate and carried through a number of refilterings, the inhibition was longer than when it was carried through successive refilterings with tumor filtrates.

Sound carrots were inoculated with a pure culture of *Bacillus carotovorus* and kept in a moist chamber until a quantity of juice from the rotting roots had collected. This was filtered, and by means of poured plates it was found to be sterile. The filtrate was added to cultures of the following organisms to test their susceptibility to it: *Bacterium tumefaciens* (hop, peach, and daisy strains), *Bacillus carotovorus*, *B. mycoides*, *B. coli*, and *B. phytophthorus*. Loop transfers were made from a 24-hour beef-bouillon culture of all but *B. mycoides*, which had too heavy a growth in 24 hours, so a 5-hour culture of it was used. Transfers were made to pH 7.4 beef bouillon, and 2, 10, and 30 drops and 2 c. c. of the filtrate were added to the cultures. The hop strain of *Bact. tumefaciens* exposed to the 2 c. c. of filtrate was the only organism that showed inhibition. This inhibition lasted only three days. The effect of the filtrate continued, however, for the hop cultures did not produce pellicles, while the hop controls and the treated peach and daisy strains all had heavy pellicles.

The effect of 30 drops and 2 c. c. of the carrot filtrate on *Bacillus carotovorus*, the organism that rotted the carrot, was to produce a

slightly heavier growth than the control in 24 hours instead of inhibiting it.

Table 4 shows the effect of the carrot filtrate on the several organisms treated with it for 24 hours. In three days there was good growth in all the cultures that had shown slight growth except the hop strain. The hop strain then had slight growth in the 30-drop and 2 c. c. filtrate cultures as well as in the 2-drop and 10-drop ones.

TABLE 4.—*Effect of adding filtrate of carrots rotted with Bacillus carotovorus to cultures of seven different organisms*

Organism	Growth 24 hours after adding—				Growth in control culture after 24 hours
	2 drops	10 drops	30 drops	2 c. c.	
Bacterium tumefaciens:					
Hop strain	Slight	Slight	Questionable	None	Good.
Peach strain	Fair	Fair	Slight	Very slight	Do.
Daisy strain	Slight	do	Good	Questionable	Do.
B. carotovorus	Fair	do	Heavier than control.	Heavier than control.	Heavy.
B. mycoides	Heavy	Heavy	Fair	Fair	Do.
B. coli	do	do	do	do	Do.
B. phytophthorus	do	do	do	do	Do.

The experiment described above was repeated with the three strains of *Bacterium tumefaciens*, more of the rotted-carrot filtrate being used. Beef-bouillon tubes, pH 7.4, were seeded with a loop of a 24-hour culture of each organism, and because of the small quantity of the filtrate, only 2, 4, and 10 drops of it were added.

The daisy and peach strains showed such slight inhibition after the third passage that they were discontinued. The hop strain, on the other hand, produced marked inhibition at once, and in 48 hours only the merest trace of growth was discernible in the 10-drop tube and a faint clouding in the 2 and 4 drop tubes, while the controls had heavy growth with pellicles. In four days the clouding in the 2, 4, and 10 drop tubes had disappeared, but two days later it returned, and a definite and continued growth followed.

While it still showed inhibition, one of the cultures was used for refiltering and exposure again to pH 7.0 beef-bouillon cultures of the hop strain, and 2, 10, 15, 20, 25, and 30 drops of filtrate were added to the fresh hop cultures. There was inhibition of growth in all for four days. Although the inhibition seemed to be a case of lysis and there was no trace of growth in the first refiltering with exposure to the organism, 1 and 2 drops of one of the inhibited cultures were added to slightly clouded fresh cultures of the hop strain. If the bacteriophage was potent, it was thought that it would clear up the clouding in these tubes. This did not occur, for there was a heavier growth in them in 24 hours. Later the inhibited culture itself became clouded.

The inhibition was more marked in the third passage than in the first and second, and freshly clouded cultures of hop strain were cleared by 10-drop and 1 c. c. additions of one of the inhibited cultures. The clearing, however, lasted for only three days, when clouding began.

The inhibition of cultures occurred likewise in the sixth and seventh passages for a few days, and during this time clouded cultures were

treated with them to see whether lysis would occur. In the sixth passage this clearing did not take place, but further growth was stopped for 3 days. In the seventh-passage tests there was a clearing in the two cultures under observation which lasted 1 and 2 days, but typical *Bacterium tumefaciens* growth began on the third and fourth days. The seventh-passage inhibited cultures were used against more clouded hop-strain cultures, and the clearing, followed later by clouding, was repeated. No lysis occurred throughout the series.

As the seventh-passage inhibited cultures were used up in testing their ability to produce lysis in the clouded cultures, a third experiment was started with a fresh lot of rotted-carrot juice which had been filtered several times. The pH of the juice was 9.6 after filtering.

Along with this series of passages of rotted-carrot filtrate and the organism, filtered juice of healthy normal carrot exposed to the hop strain of *Bacterium tumefaciens* was tested also. The passages with the two filtrates were carried with the hop strain only, and 1-day-old beef-bouillon cultures were used for seeding pH 6.7 beef-bouillon tubes to which the filtrates were added.

In the third passage of the rotted-carrot filtrate there was inhibition for 13 days and in the fourth passage for 20 days. The increase in inhibition did not continue, for it dropped to 6 days in the fifth passage, increased to 7 days in the sixth, and fell back to 6 days in the seventh, when the passages were discontinued.

The inhibition in the normal-carrot filtrate exposed to the hop strain was four days at the third passage but dropped back to two days in the fourth and fifth passages.

BACTERIOPHAGE PLAQUES

The bacteriophage plaques on beef-agar slants and plates were studied more extensively in the third experiment with the rotted-carrot filtrate. The plaques appeared on the normal-carrot filtrate plates and slants (fig. 2, A) exposed to the hop strain as well as on slants and plates poured from the rotted-carrot filtrate (fig. 3, A; fig. 4, A, C, D, E, F) exposed to the hop strain, although they were not so numerous or so well defined. In addition to these, definite bacteriophage plaques occurred on the control plates (fig. 2, B; fig. 3, B; fig. 4, B), a thing that had not occurred before in the work. Table 5 gives some of the details of the third experiment with the rotted-carrot filtrate.

The control plates and slants showed plaques only when they were made from cultures weakened by frequent transfers and grown in a medium not too favorable. Figure 4, C, illustrates an agar-slant control culture which showed no plaques, while the agar slants of the rotted-carrot filtrate, third passage with *Bacterium tumefaciens*, seeded in the same way (fig. 4, E and F) showed many.

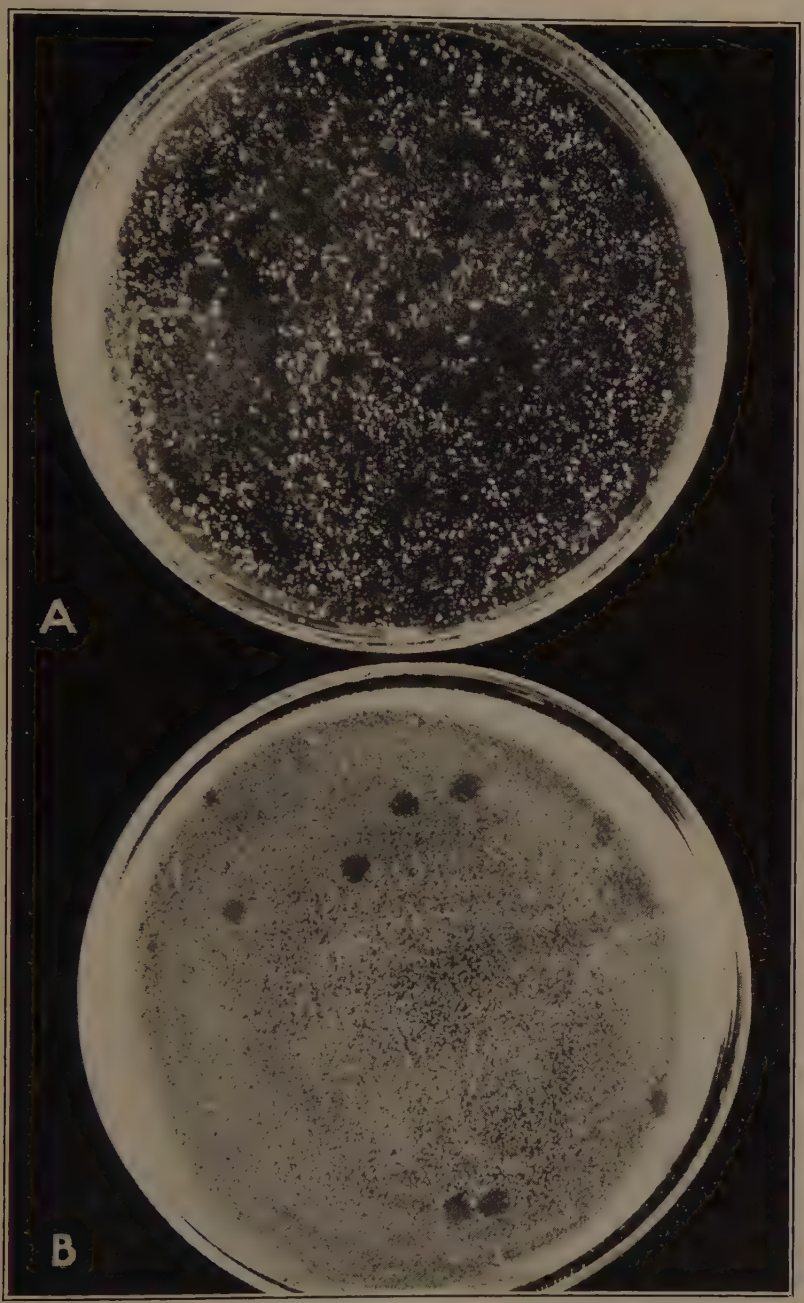

FIGURE 2.—Bacteriophage plaques on beef-agar plates: A, Sixth passage of *Bacterium tumefaciens* (hop strain) with filtrate of normal carrot; B, control, showing plaques less numerous but more striking than in A

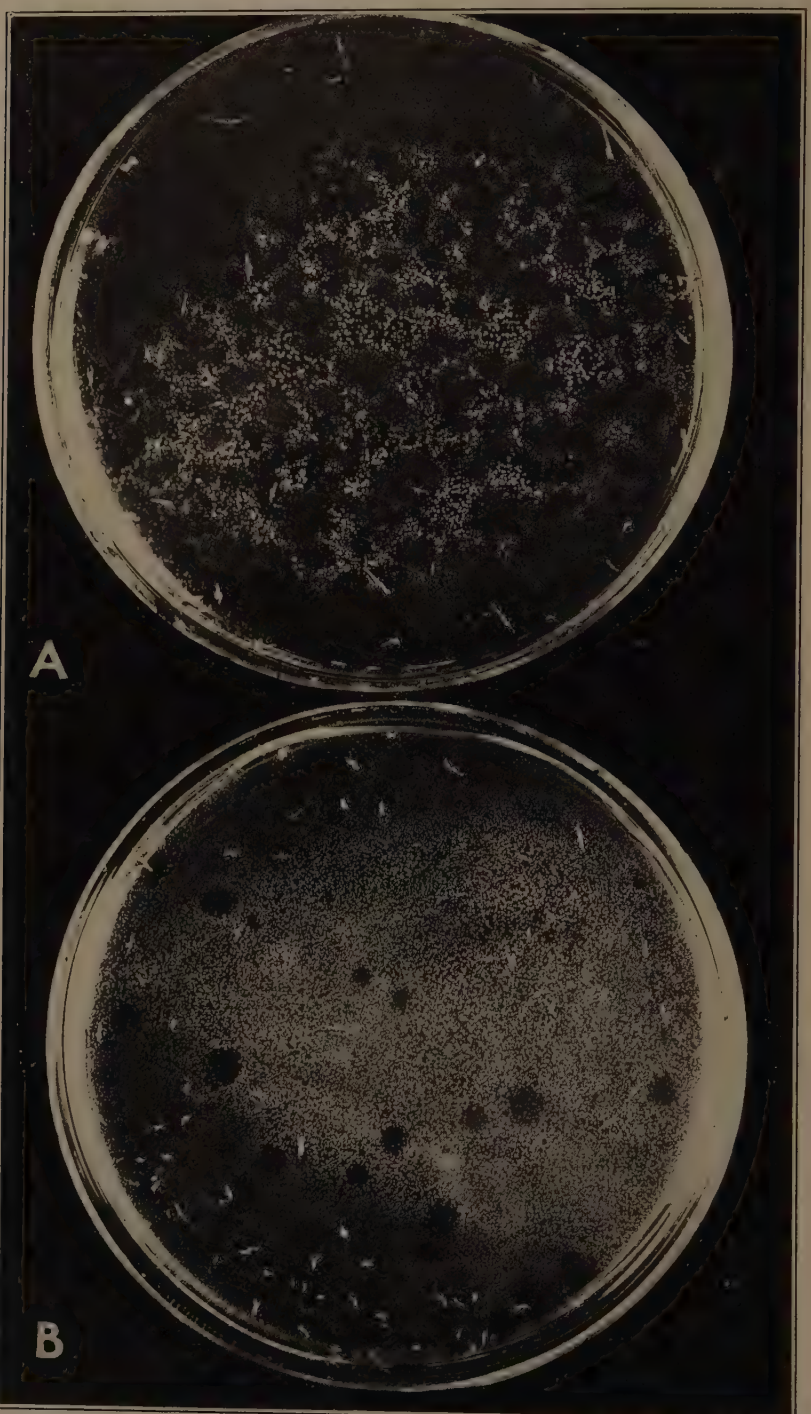

FIGURE 3.—Bacteriophage plaques on beef-agar plates with same amount of seeding: A, Fourth passage of *Bacterium tumefaciens* (hop strain) with filtrate of carrot rotted with *Bacillus carotovorus;* B, control plate of *Bact. tumefaciens* (hop strain), showing plaques also but not so numerous as in A

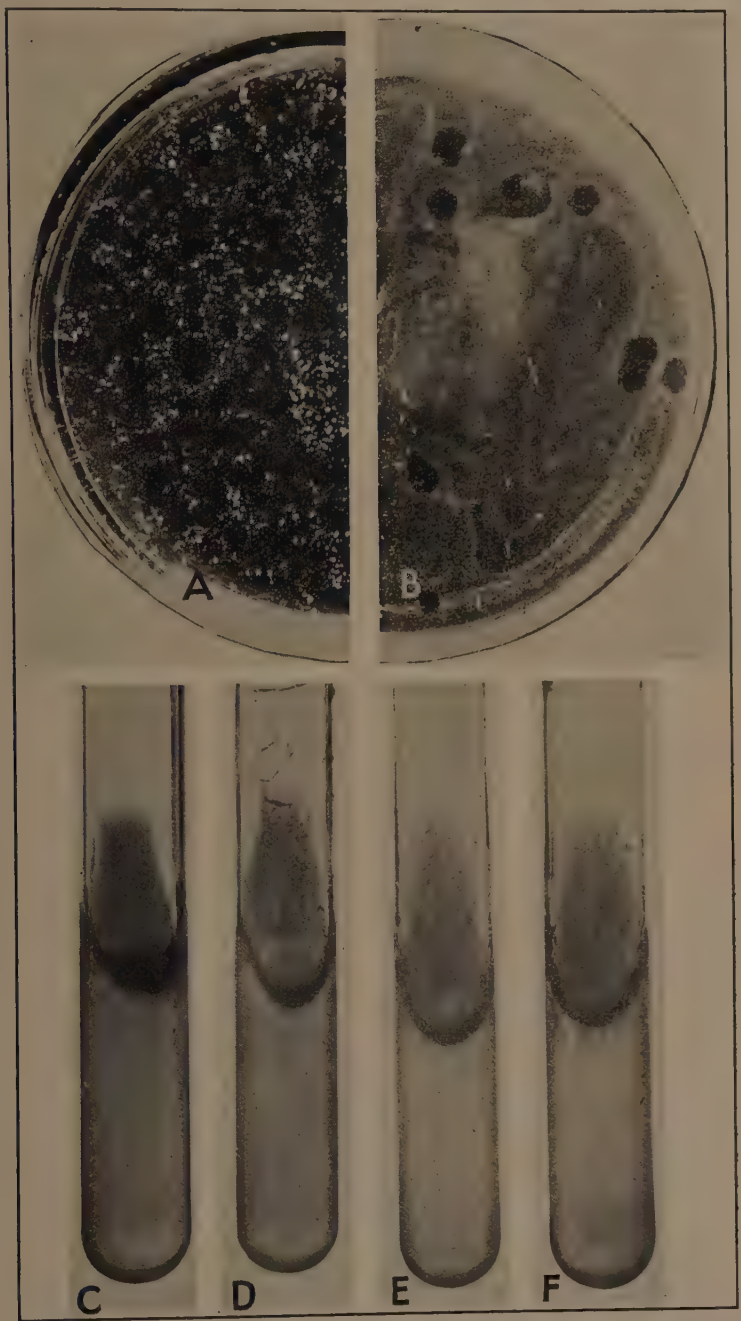

FIGURE 4.—Bacteriophage plaques of *Bacterium tumefaciens* (hop strain) on beef-agar plates and in tubes. A, Sixth passage of the organism with filtrate of rotted carrot. Note the presence of many plaques; B, Control. Note the presence of a few plaques; A and B received the same amount of seeding; C, Control culture seeded with two drops of a 24-hour beef-bouillon culture (plaques absent); D, Third passage of the organism with rotted-carrot filtrate swabbed on slant agar (tiny plaques present); E and F, Seeded with 2 drops of the third passage of the organism with the rotted-carrot filtrate (tiny plaques present)

TABLE 5.—*Effect of filtrates of rotted and normal carrots on Bacterium tumefaciens (hop strain) and the resulting bacteriophage plaques*

Passage	Filtrate of rotted carrot added to culture			Filtrate of normal carrot juice added to culture			Control culture in pH 6.7 beef bouillon	
	pH of filtrate before adding to culture, (pH 9.6 before passage with culture)	Inhibition (days)	Bacteriophage plaques	pH of filtrate before adding to culture (pH 6.8 before passage with culture)	Inhibition (days)	Bacteriophage plaques	Growth	Bacteriophage plaques
First	7.0	3	Few	7.1	1	Few	Good in 24 hours.	None.
Second	7.1	4	do	6.6	2	do	do	Do.
Third	6.8	13	More than with second passage.	6.7	4	do	do	Few.
Fourth	6.7	20	Abundant	6.7	2	Abundant	do	Moderate number; 25 on some plates.
Fifth	[a] 7.8	6	do	6.0	2	None	do	Few and inconspicuous.
Sixth	6.2	7	do	6.2	[b]	Many	[c]	12 to 15 large ones on plates.
Seventh	6.7	6	Many, but not classic type.		[b]		[c]	Some, but not classic type.

[a] Portion of filtrate stood open three days before reading was made.
[b] Notes not taken for four days; good growth then.
[c] Notes not taken for four days; heavy clouding then.

The plaques were tested in beef-bouillon cultures to see if they could produce lysis. Pieces of several of the fourth-passage plaques were cut out of the agar plates of the rotted and normal filtrates containing the organism and the control plates and were added to slightly clouded beef-bouillon cultures. The beef-bouillon did not clear, but there was no additional growth for two days, showing that the plaques had some inhibitive power. A definite clouding began on the third day or a little before that time in some cultures.

Sterile cover glasses were dropped on plaques on agar plates, removed, stained with carbol fuchsin, and studied under the microscope. The plaque picture was reproduced on the cover glass, for the tiny bacterial colonies outlining the plaques stained well. These colonies were made up mostly of swollen forms, very short rods appearing as coccus forms, and some normal-sized bacteria also, but the greater number of the normal ones were back from the margin of the plaque. A few bacteria in the center of the plaques appeared as tiny rods. These may have been drawn into the center by the removal of the cover glass from the top of agar plates, or they may have been there all the time. It is possible that they were bacteria inhibited but not destroyed by the bacteriophage. Among the swollen bacteria there were tiny particles which perhaps were the remains of swollen forms that had burst.

TESTS WITH INHIBITED CULTURES

Although the cultures of the fourth passage showed inhibition and it was thought that there was a condition of lysis, hanging drops from them were examined under the microscope 10 days after the cultures were made. Very few bacteria were located, but those found were typical rod-shaped forms of *Bacterium tumefaciens* without movement.

To study the phenomenon further, 10 drops of one of the inhibited cultures were added to slightly clouded beef-bouillon tubes. The clouding cleared somewhat in 1 day, but in 2 days definite *Bacterium tumefaciens* growth began and made slow but good development. Three days after these cultures were made the original fourth-passage cultures began to cloud, showing that there was no longer inhibition. The inhibition had lasted 20 days.

INOCULATIONS WITH INHIBITED CULTURES

The cultures inhibited 7, 13, and 20 days never developed typical *Bacterium tumefaciens* growth after growth began, but transfers from them to beef bouillon, pH 7.0, did. The clouding in the former was not heavy, no pellicle formed, and there was a precipitate of a less viscid consistency than usual at the bottom of the tube.

Ricinus plants were inoculated with the 7, 13, and 20 day inhibited cultures after growth took place in them, and also with control cultures of *Bacterium tumefaciens* made at the same time. These cultures of the sixth, third, and fourth passages, respectively, were the ones that demonstrated the bacteriophage plaques so well.

Power to infect was lost with the third passage, in which inhibition lasted 13 days, for no tumors formed (fig. 5 A), although the control cultures produced full-sized tumors (fig. 5, B).

Inoculations with the fourth passage, in which inhibition continued for 20 days, showed that some bacteria were still infectious, for tumors were produced. There was marked retardation in their development, however, for in 15 days 2 inoculations out of 5 did not show any developing outgrowth; the other three showed mere traces or swellings, while the 3 control culture tumors were 1.75 to 2 cm. in diameter. After nearly 3 months one inoculation was still negative; 2 of the tumors were only 2 cm. and 2 were 5 cm. in diameter (fig. 5, C), while the control tumors had reached their full size (5 cm.) in less than 2 months and before 3 months were necrosing. (Fig. 5, D.)

Inoculations with the sixth passage where there was inhibition for 7 days also showed a retardation of tumor formation and the presence of some infectious bacteria, as in the fourth passage. In one month two tumors of the sixth-passage inoculations were only 4 mm., while one was 12 mm., in diameter. The controls were 2 to 2.5 cm in diameter. One inoculation was negative, and at the end of three months it continued negative. The smallest tumor was 12 mm., and the largest were 2 and 4 cm. (Fig. 5, E.) The control tumors at the same time were all 5 cm. in diameter. (Fig. 5, F.)

As the plants used for the 7, 13, and 20 day inhibited-culture inoculations were of the same age, inoculated about the same time and grown under the same conditions, the difference in the time of appearance of tumors produced by these cultures (or the nonappearance

of tumors) was striking, as was also the marked difference in the sizes of the tumors within a series, irrespective of the size of the controls. The bacteria inhibited for a time by the bacteriophage and later able

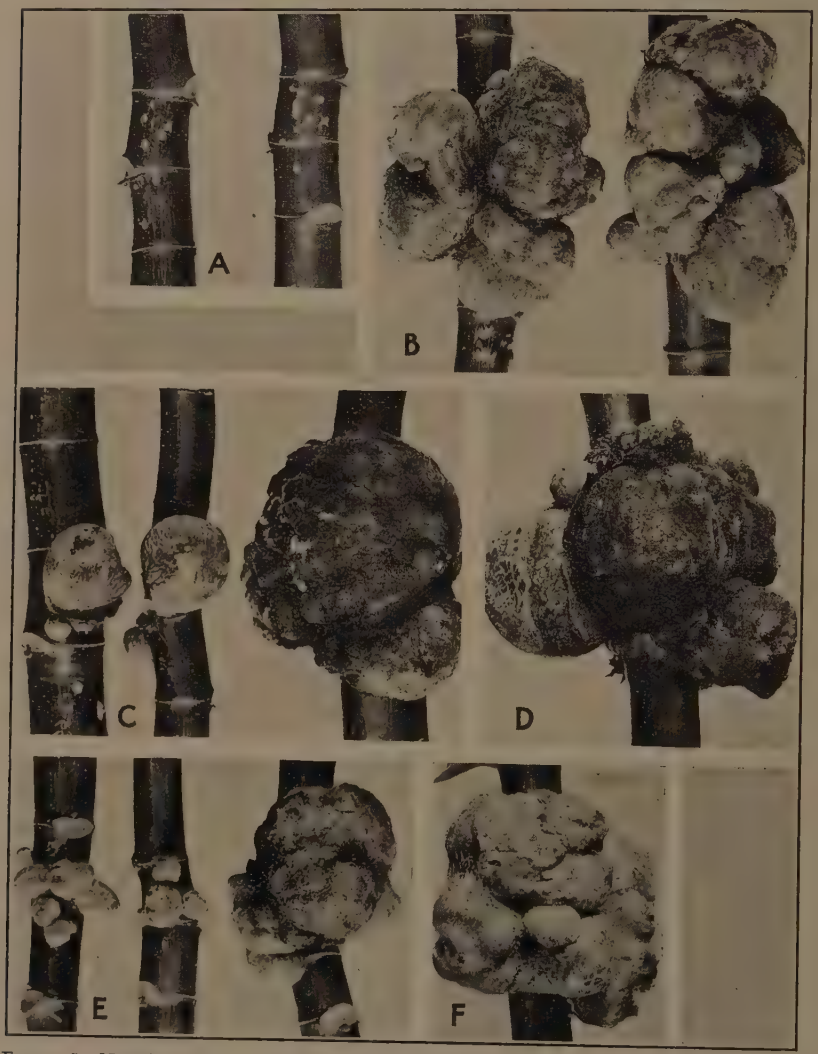

FIGURE 5.—Negative, retarded, and control infections produced by inoculating special cultures of *Bacterium tumefaciens* (hop strain) and control cultures of same organism into Ricinus plants. All reduced nearly one-half. A, Inoculation with third passage of *Bact. tumefaciens* and rotted-carrot filtrate. Photographed three months after inoculating; no infection. B, Control culture of *Bact. tumefaciens* inoculated into Ricinus plants. Photographed three months after inoculating. Regular-sized galls produced. Compare with A. C, Inoculations with fourth passage of the organism and rotted-carrot filtrate. Photographed more than three months after inoculating. Two tumors undersized and one nearly normal size. (One negative not shown.) D, Control culture inoculated into Ricinus and photographed at the same time as C. Tumor normal size. Compare with C. E, Inoculations with sixth passage of organism and rotted-carrot filtrate. Photographed three months after inoculating. Two tumors undersized and one nearly normal. (One negative not shown.) F, Control culture inoculated into Ricinus and photographed at the same time as E. Tumor normal size. Compare with E

to overcome its action do not seem to retain their infectious ability to the same degree. A young normal culture of the hop strain of *Bacterium tumefaciens* inoculated into Ricinus gave 100 per cent

infection, the tumors varying but little in size when conditions were similar. The size of mature tumors produced by a normal culture of hop strain on Ricinus depended on the age of the plants at the time of inoculation, the time of year inoculated, and the rapidity with which the plants were grown. The size at maturity may range from 1.75 to 6 cm. Table 6 shows the results of the inoculations with the third, fourth, and sixth passage inhibited cultures after growth had taken place in the cultures.

TABLE 6.—*Results of inoculating Ricinus plants with equal-aged inhibited and control cultures of Bacterium tumefaciens*

Description of culture	Time after inoculation	Inoculation with inhibited cultures			Inoculation with control cultures		
		Plants inoculated	Tumors produced	Diameter of of tumors	Plants inoculated	Tumors produced	Diameter of tumors
		Number	*Number*	*(Mm.)*	*Number*	*Number*	*Mm.*
Third passage of Bacterium tumefaciens with rotted carrot filtrate in which growth did not begin until after 13 days of inhibition.	1 month	4	0		3	3	20–25
	2 months	4	0		3	3	35–40
	3 months	4	0		3	3	45–50
Fourth passage in which growth was inhibited 20 days.	10 days	5	0		3	3	4–5
	15 days	5	3	2–3	3	3	17–20
	24 days	5	3	3, 4 and 12	3	3	40–45
	1 month, 17 days.	5	[a] 3	3, 20, and 25	3	3	50
	2 months, 23 days.	5	4	20 and 50	3	3	[b]
	3 months, 15 days.	5	4	25 and 50	3	3	[c]
Sixth passage in which growth was inhibited 7 days.	1 week	4	0		3	0	
	1 month	4	3	4 and 12	3	3	20–25
	2 months, 4 days.	4	3	6, 12, and 25	3	3	45–50
	3 months	4	3	12, 20, and 40	3	3	50

[a] Also one starting to swell. [b] Beginning to necrose. [c] Badly necrosed.

REPETITION OF D'HÉRELLE AND PEYRE'S EXPERIMENT IN WHICH THEY PRODUCED TUMORS BY A FILTERING FORM OF BACTERIUM TUMEFACIENS

In 1927 D'Hérelle and Peyre (*4*) published a report of some of their work with plant tumors produced by *Bacterium tumefaciens*. They believe that there are two kinds of colonies isolated from the tumor, one an ultrapure colony, nonparasitic, and another contaminated by the bacteriophage, which is the infectious colony. They believe also that the infection of the bacteria by the bacteriophage produces invisible forms of the bacteria, which they call protobacterial forms; and with the filtrate from plant tumors they have been able to obtain the return of the filtering protobacterial form to the normal bacterial form. Inasmuch as the microscopic examination of tumors does not show any bacteria, they suggest that the true parasite is the protobacterial form of the organism. These protobacterial forms they believe can act as virus filtrates and when inoculated into susceptible plants produce tumors. Their experiments with a filtrate from sugar-beet tumors induced by inoculating with *Bacterium tumefaciens* bore out this belief. Out of 20 sugar beets inoculated with the filtrate, 14 produced tumors within 70 days, and platings from 6 of the tumors gave typical cultures of *Bacterium tumefaciens*. According to D'Hé-

relle's hypothesis, the relation of the bacteria parasitized by the bacteriophage is a symbiotic one. This symbiosis has become stronger through continued passages through living hosts, and it is very difficult to break up, sometimes impossible with the methods now known. .

The writers carried out the same experiment using Ricinus tumors for filtering. As soon as the filtrate was obtained, it was inoculated into 40 Ricinus plants with a hypodermic needle. The plants were kept under observation for several months, but no tumor appeared. The experiment was made three times and with three different lots of tumors. Each time other Ricinus plants of the same age were inoculated with a culture of *Bacterium tumefaciens* and held as controls. These control cultures always produced sizable tumors.

Poured plates of the three filtrates were made and studied for sterility. No colonies of *Bacterium tumefaciens* appeared. However, nine other colonies of several types were observed on the three sets, and, in the belief that these might be of value should there be a protobacterial form, they were transferred, cultured, and inoculated into Ricinus plants. No tumor arose from these vagrant colonies. In all probability they were air colonies that had no connection with the filtrates, and the filtrates themselves were free from *Bacterium tumefaciens*, as the hypodermic inoculations showed.

In order to test further the presence of an infectious filtering form of *Bacterium tumefaciens*, flasks of beef bouillon, pH 7.0, were inoculated with the hop strain, left to grow for 3 days, and then filtered. Sixteen Ricinus plants were inoculated hypodermically with some of the filtrate, and other Ricinus plants of the same age were inoculated with a 3-day-old control culture of the hop strain. Plates were poured from some of the filtrate to test its sterility. The plates were examined during a period of 11 days, but no colonies of *Bact. tumefaciens* appeared. The three colonies that came up on the plates were transferred, and inoculations into Ricinus were made with subcultures. The inoculations with both the sterile filtrate and the three suspicious colonies were kept under observation more than 2 months, but there was no trace of outgrowths. The control culture inoculations produced large tumors.

Part of the sterile beef-bouillon filtrate was held in the laboratory to give an opportunity for the filtering bacterial form to develop if such form occurs. There was a clouding in the filtrate in four days, but it was not a typical *Bacterium tumefaciens* growth. However, inoculations into Ricinus plants were made with the clouded filtrate because of the possibility that the growth might be a filtering infectious form of *Bact. tumefaciens* as D'Hérelle and Peyre assert. These inoculations were watched for two and one-half months, but no trace of tumor growth occurred.

DISCUSSION

The conditions required for obtaining a culture of *Bacterium tumefaciens* that will produce more quickly growing and larger tumors than the controls can be brought about readily by adding a highly diluted tumor filtrate to a young culture of the organism and letting it grow two to four days before inoculating with it. There is some evidence

that filtrates of normal-plant juices also have the ability to produce more rapidly growing and larger tumors than the controls, although this evidence is based on experiments with normal Ricinus and normal carrot filtrates only.

A sewage filtrate combined with the organism produced faster growing and larger tumors than a filtrate of a sugar-beet tumor combined in the same way. The latter in turn produced more rapidly growing and larger tumors than the controls. A filtrate to which the organism was exposed as soon as possible after the tumor was crushed and filtered gave a culture which seemed to be nearly as effective in producing the rapid-growing and oversized tumors as a filtrate which had oxidized several days.

It may be that the addition of the fresh filtrate cements the union of the bacteria and the bacteriophage more closely and a higher degree of virulence is temporarily established.

No absolute lysis was produced in these experiments. It is quite possible, however, that the apple or rose strains of *Bacterium tumefaciens* might be cultured with some tumor filtrate and produce it. These two show evidence of being about the weakest strains of *Bact. tumefaciens*. This weakness, which manifests itself in producing tumors slowly when these strains are inoculated into susceptible plants, even into their native hosts, and the inability of these strains to produce tumors in some of the common plants easily grown in greenhouses, kept the writers from pursuing the development of lysis with the apple and rose strains through successive passages with a filtrate. The writers are assuming that the weakness of the strains is linked with the more facile separation of the bacteriophage from the organism. Very young cultures of the organism seemed to be necessary for the passages with the filtrates to produce inhibition. To obtain young cultures frequent transfers were required, which apparently had a tendency to bring about an instability of the close union that had become established between the bacteriophage and the organism and that allowed a release of the bacteriophage. In this temporary release the bacteriophage could affect the organism by its own power to cause inhibition. This release is manifested also by means of the bacteriophage plaques on agar slants and plates, and if a high potency has been reached, by marked inhibition. No doubt a condition could be brought about where there would be complete lysis. The culture that was inhibited for 13 days did not produce tumors when inoculated into Ricinus plants, although it was not used for inoculations until a few days after growth appeared. The control cultures of the same age produced tumors. An inhibited culture of 20 days produced tumors, retarded in appearing, but nevertheless present. The organisms resistant to the bacteriophage in the latter case were evidently of an infectious nature, while in the former they were not.

The bacteriophage plaques appearing on the plates poured from a pure culture of the hop strain of *Bacterium tumefaciens* as well as on those poured from filtrate *Bact. tumefaciens* cultures indicate that the lytic or inhibiting principle is carried along with the growth of the organisms irrespective of any active filtrate. The plaques from a pure culture were large and distinct but never so numerous as those on the filtrate-culture plates.

Sometimes there was a tumor-juice filtrate carried in passages with *Bacterium tumefaciens* which seemed not to activate the young culture, and if there was no inhibition of the organism in the early passages, it did not appear in a later passage. Whether the age of the tumor, the host of the tumor, or the rapid or slow rate at which the tumors had developed played a part in the presence and potency of the bacteriophage in the extracted juices was not fully determined. Frequently there was some evidence for thinking so. For instance, a sterile filtrate of old Ricinus tumors with necrosed areas gave no indication of inhibition when *Bact. tumefaciens* was exposed to it with three refilterings, nor at the beginning did it give evidence of producing acceleration of growth. The filtrates of very young tumors were not found satisfactory to work with. Whether this was because of the age or because the bacteriophage adhered to the more colloidal juice and did not pass through the filter is not known. It was found more difficult to filter the juice extracted from very young tumors. Those tumors not yet mature were found quite satisfactory and their filtrates could produce both inhibition and acceleration of growth when *Bact. tumefaciens* was exposed to them.

SUMMARY

The potentials of juices of normal and tumor tissue and *Bacterium tumefaciens* in culture revealed the following facts: (1) The pH of the freshly extracted juice of tumor tissue was always higher, that is, the reaction was more alkaline than that of the normal tissue; (2) the total acid content was greater in the tumor juice than in the normal juice; (3) the oxidation potential was always greater (more negative) in the freshly extracted tumor juice than in the normal juice—that is, the electrical charge carried by the gold electrodes in the fresh tumor juice was always negative (−) to that in the normal juice; (4) the electrical charges (accompanied always by a change in pH) on the gold electrodes were exactly reversed in the juices of the normal and tumor tissues upon further oxidation; (5) the change in pH produced by the growth of the organisms *Bact. tumefaciens* in beef-bouillon culture and *Bacillus carotovorus* inoculated into fresh carrots, progressed in the same direction (pH increase) as the change of pH of the normal juice upon further oxidation rather than in the direction (pH decrease) of the pH changes of the tumor juices; (6) the presence of a bacteriophage was established.

The presence or absence of plaques on poured plates appears to be correlated with the rise and fall of the pH of the plant filtrate or the pH of the culture of *Bacterium tumefaciens* treated with the filtrate. The pH value of the *Bact. tumefaciens* cultures which produced plaques when only 24 hours old was 6.7, and that of a normal *Bact. tumefaciens* culture 1 month old was 8.2. No plaques were present. A *Bact. tumefaciens* culture treated with a *Bacillus carotovorus* filtrate of pH 9.6 after 24 hours was pH 7.1 and after 4 days was pH 6.6, or slightly more acid than the fresh normal culture. This culture treated with *B. carotovorus* filtrate produced a greater number of plaques than *Bact. tumefaciens* cultures treated with other filtrates such as normal carrot, Ricinus tumor, or *Bact. tumefaciens*.

Accelerated growth of bacteria with increased pathogenicity seems to be associated with the phenomenon of bacteriophagy as well as does the retarded or inhibited growth of bacteria, causing either decreased or delayed pathogenicity or none at all.

The accelerated growth of the bacteria and increased size of tumors as compared with those of the controls were induced by the addition of high dilutions of tumor filtrates to young cultures used later for the inoculations. There is some evidence that filtrates of normal plant juices likewise have the ability to produce larger and more rapidly growing tumors than the controls. Inhibition of the bacteria from 4 to 20 days was obtained by successive passages of the tumor filtrate and light suspensions of the bacteria through Chamberland L3 filters. Tumors of small size or none at all were obtained through inoculations with these inhibited cultures after growth took place in them. Slight dilutions of the tumor filtrate grown with the culture inhibited the growth of bacteria and retarded tumor development. Tumors produced by these inhibited cultures occasionally reached the size of the control tumors, after a long slow growth. Complete lysis, the dissolving of all the bacteria in a culture, did not take place in the course of the work, which continued more than a year.

Bacteriophage plaques were obtained from the plant-tumor filtrates combined with *Bacterium tumefaciens*. The greatest number and largest ones, however, were obtained on beef-agar plates and slants from the rotted-carrot filtrate to which *Bact. tumefaciens* (hop strain) was exposed.

Typical bacteriophage plaques were obtained also on beef-agar plates and slants from a pure culture of *Bacterium tumefaciens*, and also in plates poured from a normal-carrot filtrate combined with *Bact. tumefaciens*. These plaques were not so numerous as those on the rotted-carrot filtrate plates.

D'Hérelle and Peyre assert that there exists a bacterial filtering form of *Bacterium tumefaciens* which is the infectious principle and which can be cultivated back to the nonfiltering form. Three repetitions of their experiment with sterile filtrates of *Bact. tumefaciens* tumors and also one with a sterile filtrate of beef-bouillon cultures of the organism were carried out, but the results were negative.

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